Volume 180, number 2 FEBS 2200 January 1985

An electrogenic uniport mediates light-dependent Ca^{2+} influx into intact spinach chloroplasts

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Received 19 November 1984

Light-dependent Ca^{2+} influx into intact spinach chloroplasts, measured with the metallochromic indicator arsenazo III, is stimulated by uncouplers (FCCP, CCCP, nigericin) and inhibited by ruthenium red. The data presented demonstrate that light-dependent Ca^{2+} influx into chloroplasts is electrogenic and mediated by a uniport-type carrier. The characteristics of the carrier system are similar to those of the Ca^{2+} uniport of mitochondria.

Spinach *Chloroplast* *Ca^{2+}* *Electrogenic uptake* *Uniport*

1. INTRODUCTION

Calcium exhibits specific functions in the regulation of growth, development, and metabolism in plants [1,2]. It may affect cellular processes either directly or through the Ca^{2+}-calmodulin regulatory system [1–4]. Light mediates the regulation of several chloroplast enzymes, some of which are Ca^{2+}-dependent enzymes [3,4]. In addition, the existence of specific Ca^{2+}-binding sites at photosystem II [5,6] and the involvement of Ca^{2+} in the spillover-phenomenon [7] suggest an important role for calcium within the chloroplast. Recently it has been shown that Ca^{2+} influx into wheat and spinach chloroplasts is light-dependent [8,9]. In this study we characterize the light-dependent Ca^{2+} influx into intact spinach chloroplasts as an electrogenic process mediated by a uniport-type carrier system.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; $\Delta\bar{\mu}_{H^+}$ proton motive force; ΔE, membrane potential; ΔpH, transmembrane pH gradient; PGA, 3-phosphoglyceric acid

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Percoll was obtained from Pharmacia; arsenazo III, valinomycin, nigericin, CCCP, and ATP from Sigma; FCCP from Serva; vanadate from EGA-Chemie, and Chelex-100–200 mesh was from Bio-Rad. Spinach calmodulin was kindly provided by M. Burchert (this laboratory). All other chemicals were from Merck and of analytical grade.

2.2. *Preparation and purification of spinach chloroplasts*

Spinach was grown as in [10]. Chloroplasts were isolated as in [11] except that the resuspension medium contained 330 mM sorbitol, 1 mM $MgCl_2$ and 50 mM Hepes–KOH (pH 7.0). For further purification discontinuous percoll gradients were used. The gradient medium contained 330 mM sorbitol, 20 mM KCl, 20 mM Hepes–Tris (pH 7.0). In 12 ml tubes 4.5 ml of chloroplast suspension was layered on top of 1.5 ml 65% (v/v) and 4 ml 40% percoll and centrifuged for 3 min at 1500 × *g*. Intact chloroplasts were collected with a Büchler Auto-Densi-Flow II gradient fractionator, diluted with an equal volume of resuspension medium and pelleted by a 50 s centrifugation at 750 × g to remove the percoll. The pellet was resuspended in

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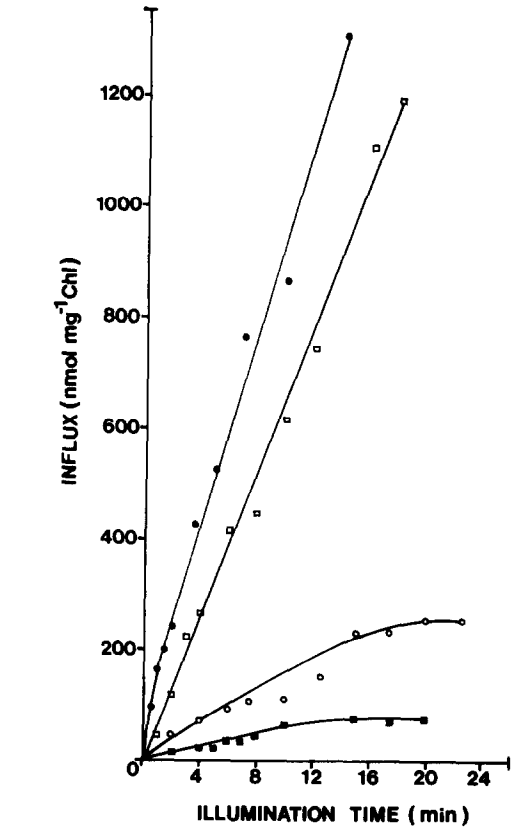
00145793/85/$3.30

a medium (treated with Chelex-100) which contained 330 mM sorbitol, 20 mM Hepes-Tris (pH 7.0), 20 mM KCl and 1 mM $MgCl_2$ (the latter added after Chelex-100 treatment). Purity of the chloroplast suspension was evaluated by (a) electron microscopy, and (b) the marker enzymes catalase [12], cytochrome-*c*-oxidase [13], nitrate reductase [14], and NADH-cytochrome-*c*-reductase [15]. No contaminating organelles were detected, and contamination with cytoplasmic enzymes was negligible (e.g., nitrate reductase: 3%). Intactness of chloroplasts was measured as in [16] and was always ≥ 90%. PGA (0.5 mM)-dependent oxygen evolution was measured according to [17], but NaCl and catalase were omitted. Chlorophyll was determined as in [18].

2.3. *Measurement of Ca^{2+} influx*

Arsenazo III was purified as in [19]. The standard assay contained 330 mM sorbitol, 20 mM Hepes-Tris (pH 7.0), 20 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.5 mM PGA, 50 μM arsenazo III, 50–60 μM $CaCl_2$, and 10–20 μg chlorophyll. Sorbitol, Hepes and KCl solutions were treated with Chelex-100 to reduce the Ca^{2+} content. The extinction changes were followed with a double beam spectrophotometer (Sigma ZWS II) at 660–690 nm according to [20]. The standard conditions were 25°C, 3 min preincubation in the dark with the different additions, then illumination with 85 $W \cdot m^{-2}$ in a stirred and cooled cuvette. As a control the same assay was done in the presence of 100 μM EGTA without $CaCl_2$. The measured extinction change of the control was subtracted from that measured for the assay (without EGTA). In all experiments, except the time-course experiment, Ca^{2+} influx/efflux was measured after a 2 min period of illumination. Internal calibrations were done as in [20] for each different set of conditions. Different free Ca^{2+} concentrations were obtained using EGTA buffers.



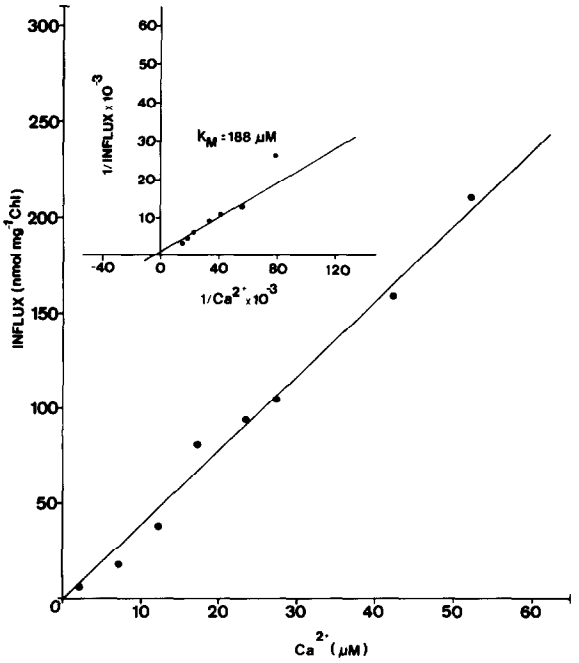


Fig. 1.(a) Time course of light-dependent Ca^{2+} influx into intact spinach chloroplasts at different external Ca^{2+} concentrations. Assay conditions as in section 2.3. 50 μM free Ca^{2+} (•), 20 μM free Ca^{2+} (□), 10 μM free Ca^{2+} (○), 5 μM free Ca^{2+} (■). (b) Effect of different Ca^{2+} concentrations on light-dependent Ca^{2+} influx into intact spinach chloroplasts. Assay conditions as in section 2.3.

3. RESULTS

Under our assay conditions a light-dependent Ca^{2+} influx into intact spinach chloroplasts is observed (fig. 1a). The rate and extent of Ca^{2+} influx is dependent on the concentration of free Ca^{2+} in the assay medium and the duration of illumination. The net influx is saturated after 10 min in the presence of 5 μM free Ca^{2+}, and after 15 min in the presence of 10 μM free Ca^{2+} in the assay medium. At these two Ca^{2+} concentrations the maximum amount of Ca^{2+} uptake by the chloroplasts is 80 and 260 nmol·mg^{-1} Chl, respectively, which corresponds to 21 and 43% of the total available Ca^{2+} in the medium. The observed Ca^{2+} influx is not due to chloroplast damage during the assay: in an average experiment chloroplasts were 98% intact before the assay and 96% intact after the assay. Light-dependent Ca^{2+} influx is completely abolished by treatment with heat (5 min, 100°C) or Triton X-100 (0.25 vol.%). In addition, no Ca^{2+} influx is detected after chemical fixation of chloroplasts with 5% glutaraldehyde. Photosynthetic activity of the chloroplasts was measured by PGA-dependent oxygen evolution. The observed rates are identical to that measured using the assay medium of [17]. Ca^{2+} influx is linear between 0 and 60 μM free Ca^{2+} (fig. 1b). The apparent k_M is 188 μM (fig. 1b). The average rate of Ca^{2+} influx under the conditions described in section 2.3. is 6.6 μmol·mg^{-1}·h^{-1} ($n = 32$ different chloroplast preparations).

The light-dependent Ca^{2+} influx is observed at relatively low light intensities. Half-maximum influx is achieved at 40–50 W·m^{-2}. DCMU completely inhibits Ca^{2+} influx at concentrations > 1 uM. Spinach calmodulin (4.8 μg·ml^{-1}) and vanadate had no effect on the light-dependent Ca^{2+} influx (detailed results not shown). Low concentrations of FCCP and CCCP, which are sufficient to uncouple electron transport, stimulate Ca^{2+} influx, whereas higher concentrations, known to inhibit electron transport [21], suppress Ca^{2+} influx (fig. 2). The uncoupling K^+/H^+ exchanger nigericin also enhances Ca^{2+} influx (table 1). Additional KCl (15–30 mM) given 3 min before illumination stimulates Ca^{2+} influx (table 1). At standard concentrations of KCl (20 mM) in the assay medium Ca^{2+} influx increase following the addition of valinomycin (table 1). When an additional 30 mM KCl are added, valinomycin suppresses Ca^{2+} influx and in several experiments even caused Ca^{2+} efflux (table 1). Nigericin abolishes the Ca^{2+} efflux induced by valinomycin in the presence of high external KCl concentrations (table 1).

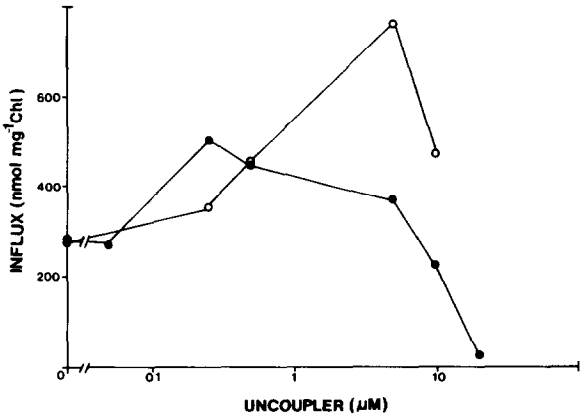


Fig. 2. Effect of uncouplers on light-dependent Ca^{2+} influx into spinach chloroplasts. Assay conditions as in section 2.3. FCCP (•), CCCP (○).

Ruthenium red inhibits light-dependent Ca^{2+} influx into spinach chloroplasts (fig. 3). At concentrations of ruthenium red (≤ 10 μM) which significantly inhibit Ca^{2+} influx, PGA-dependent

Table 1

Effect of valinomycin, nigericin, and combinations of both in relation to external KCl concentrations on light-dependent Ca^{2+} influx into intact spinach chloroplasts

KCl, added to the the medium (mM)	Valinomycin (μM)	Nigericin (μM)	Ca^{2+} influx (nmol·mg^{-1} Chl)
0	0	0	48.8
15	0	0	213.3
30	0	0	246.8
0	0	0.5	195
0	0.5	0	106.7
0	0.5	0.5	280.4
30	0	0.5	383.9
30	0.5	0	-97.5
30	0.5	0.5	414.4

Assay conditions as in section 2.3.

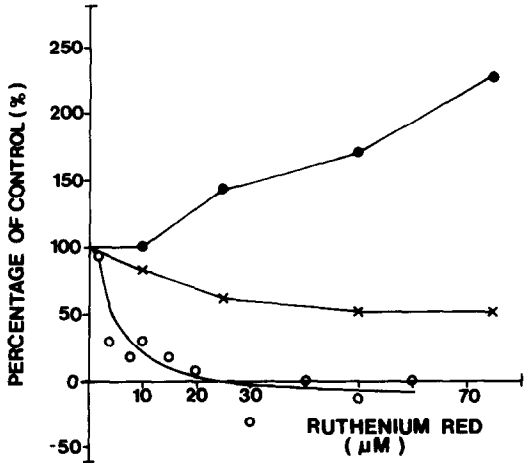


Fig. 3. Inhibition of light-dependent Ca^{2+} influx into spinach chloroplasts by ruthenium red. Assay conditions as in section 2.3. Ca^{2+} influx/efflux (○), PGA-dependent oxygen evolution (×), ferricyanide-dependent oxygen evolution by broken chloroplasts (•).

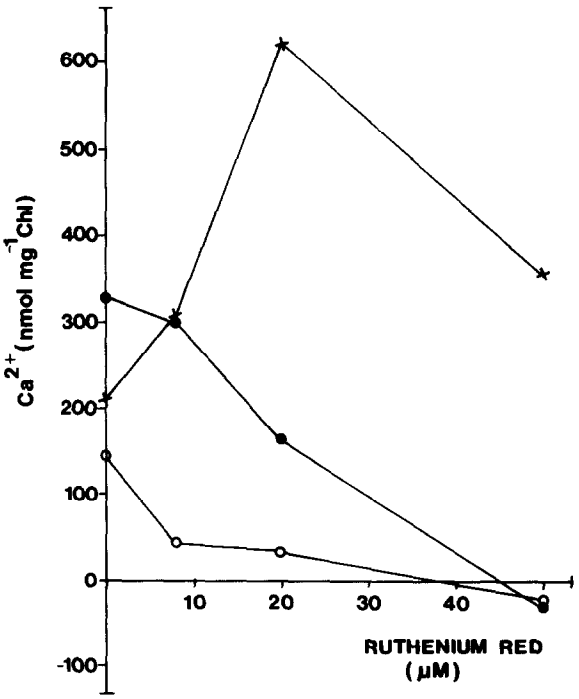


Fig. 4. Effect of ruthenium red on normal, FCCP- and ionophore A 23187-enhanced Ca^{2+} influx into spinach chloroplasts. Assay conditions as in section 2.3. Control (○), 0.5 μM FCCP (•), 10 μM A 23187 (×).

oxygen evolution is only slightly affected (fig. 3). To exclude the possibility that the effect of ruthenium red is due to unspecific charge-interactions at the envelope, we tested the ability of ruthenium red to inhibit the enhanced Ca^{2+} influx mediated by uncouplers and the Ca^{2+}-ionophore A 23187. FCCP-enhanced Ca^{2+} influx is inhibited by ruthenium red, while A 23187-mediated Ca^{2+} influx is not inhibited by it (fig. 4).

4. DISCUSSION

4.1. *Transport mechanism*

The observations made in this study indicate that ATP is not directly involved in light-dependent Ca^{2+} influx into intact spinach chloroplasts because: (i) Ca^{2+} influx is insensitive to vanadate, a potent inhibitor of ion transport ATPases [22], (ii) Ca^{2+} influx is enhanced by uncouplers, and (iii) calmodulin, which stimulates the plasma membrane Ca^{2+}-ATPase (23), has no effect on Ca^{2+} influx into spinach chloroplasts.

Ruthenium red is known as an inhibitor of electrogenic Ca^{2+} influx mediated by uniport-type carriers [24–27]. We can exclude unspecific effects of ruthenium red, since at inhibitory ruthenium red concentrations electron transport is only slightly affected. In addition, unspecific charge interactions of ruthenium red at the chloroplast envelope can be dismissed, since A 23187-mediated Ca^{2+} influx could not be inhibited by ruthenium red [26].

Two criteria have been used to distinguish between Ca^{2+} influx mediated by a uniport-type carrier and that mediated by a Ca^{2+}/H^{+}-exchange carrier [27]: uniport-type influx is sensitive to ruthenium red and enhanced by uncouplers. These criteria for a uniport-type carrier are met by the light-dependent Ca^{2+} influx into spinach chloroplasts.

Protons might act as the counterions to Ca^{2+} influx [8] through the uniport, since they are extruded by the chloroplast in the light [17] and their amount would be sufficient to balance Ca^{2+} influx.

4.2. *The driving force*

The inhibition of light-dependent Ca^{2+} influx by DCMU indicates that $\Delta\tilde{\mu}_{H^+}$ (established by electron transport) most likely triggers Ca^{2+} influx. The ΔpH component is not involved, since Ca^{2+} influx can be stimulated by different types of un-

couplers. Thus ΔE is presumably the driving force, since the ΔE-modulating K^+-ionophore valinomycin stimulates Ca^{2+} influx at low K^+ levels (ΔE is high), and inhibits Ca^{2+} influx at high K^+ levels (ΔE is low). The effect of nigericin is probably due to its uncoupling activity which promotes Ca^{2+} influx.

4.3. *Comparison with mitochondrial Ca^{2+} influx systems*

Ca^{2+} influx into animal [25,27] and plant mitochondria [23,24] is mediated by an electrogenic uniport-carrier system similar to that described here for chloroplasts. The influx is sensitive to ruthenium red [24–27] and has been shown to be under the control of ΔE [28,29].

4.4. *Rates and affinity*

The measured Ca^{2+} influx rate of 6.6 $\mu mol \cdot mg^{-1}$ Chl $\cdot h^{-1}$ for spinach chloroplasts is in agreement with rates measured for chloroplasts in other studies [8,30]. On a protein basis the chloroplastic Ca^{2+} influx rates are similar to those of plant [31] and rat liver mitochondria [27]. From a comparison of k_m values it has been concluded that the affinity of the mitochondrial system is at least 10-fold lower than the affinity of the plasma membrane system [23]. However, a comparison of the apparent first-order rate constants (V_{max}/K_m), which reflect the affinity of an enzyme to its substrate at very low substrate concentrations [32], shows that all three Ca^{2+}-transport systems (plasma membrane, chloroplasts, mitochondria) have similar affinities towards Ca^{2+} at very low Ca^{2+}-levels.

4.5. *Physiological implications*

We can calculate from the maximum amount of Ca^{2+} influx, assuming a chloroplast volume of 26 μl [33], that the calcium concentration inside the chloroplast increases by 3–9 mM when 5–10 μM free Ca^{2+} is present in the assay medium. The actual concentration of free Ca^{2+} inside the chloroplast would, however, be much less. Targets for chloroplastic Ca^{2+} include light-activated, Ca^{2+}-regulated enzymes (discussion in [3,4,34, 35]). Other possible sites of action include various photochemical reactions [5–7]. We suggest that the Ca^{2+}-carrier system of the chloroplast-envelope may not only be important for regulation of Ca^{2+}-dependent processes inside the chloroplast, but it could also affect the Ca^{2+}-level in the cytosol and therefore exert some influence on other Ca^{2+}-regulated cellular functions in plants.

ACKNOWLEDGEMENTS

The authors are indebted to Dr Joe Holtum for useful discussions. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

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